Long-Term Protection of Hematopoiesis Against the Cytotoxic Effects of Multiple Doses of Nitrosourea by Retrovirus-Mediated Expression of Human O\(^{6}\)-Alkylguanine-DNA-Alkyltransferase

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A human \(O^{6}\)-alkylguanine-DNA-alkyltransferase (ATase) cDNA-containing retrovirus was used to infect murine long-term primary bone marrow cultures. High levels of ATase expression were obtained, and colony-forming cells of the granulocyte-macrophage lineage from the cultures transduced with the human ATase retrovirus were three times more resistant to the alkylating agent, \(N\)-methyl-\(N\)-nitrosourea (MNU), than control cultures. Furthermore, expression of the human ATase protected long-term hematopoiesis, measured as the output of progenitor cells to the nonadherent fraction of the culture, against the cytotoxic effects of repeated exposures to MNU. These results clearly show that a human ATase cDNA-containing retrovirus can be used to infect long-term primary bone marrow cultures and that this attenuates their sensitivity to nitrosoureas. © 1996 by The American Society of Hematology.

METHYLATING AGENTS (such as procarbazine and dacarbazine) and chloroethylyating agents (such as streptozotocin, bischloroethyl-nitrosourea [BCNU] and other nitrosoureas) are frequently used singly or in combination with other cytotoxic agents in the treatment of lymphomas and various solid tumors. The major DNA lesion responsible for the toxic effects of the methylating and chloroethylating agents is \(O^{6}\)-alkylguanine.\(^1\)\(^,\)\(^2\) Methylation of the \(O^{6}\)-position of guanine is thought to be cytotoxic because it causes the misincorporation of thymine on DNA replication: the resulting \(O^{6}\)-methylguanine (\(O^{6}\)-meG)-thymine base pair can be a substrate for a mismatch correction system. However, the newly synthesized strand is incised and this leads to the resynthesis of the \(O^{6}\)-meG-thymine pair, a cycling process that presumably results in loss of cell viability. The cytotoxic effects of the chloroethylyating agents are mediated by the formation of \(O^{2}\)-chboroethylguanine, which gives rise to DNA interstrand cytosine-guanine crosslinks via an intermediate cyclic \(N^{2}\)-\(O^{2}\)-ethano derivative. The coding properties of \(O^{6}\)-alkylguanine may also result in G:C to A:T transition mutations as are frequently found in activated oncogenes such as \(ras\).\(^3\) \(O^{6}\)-alkylation of guanine can be corrected by a specific DNA-repair protein \(O^{6}\)-alkylguanine-DNA-alkyltransferase (ATase), which transfers the alkyl group from guanine to a cysteine residue at the active site of the ATase, a process that leads to the inactivation of the repair protein.\(^1\)\(^,\)\(^3\)

The main limitation in the clinical use of these alkylating agents is their dose-related acute toxicity to the hematopoietic system.\(^4\) In addition, presumably because of their mutagenic potential, they also carry the risk of inducing acute myeloid leukemia in patients surviving for long periods after successful therapy of the primary tumor.\(^5\) One of the possible approaches to these problems is to introduce drug-resistance genes into the bone marrow (BM) to protect normal hematopoietic cells from chemotherapy-related toxicities.

Hematopoietic cells, particularly those of human origin,\(^5\)\(^,\)\(^6\) generally express low levels of ATase and it is not unreasonable to suggest that this is the basis of their high sensitivity to the toxic effects of the nitrosoureas and related alkylating agents.\(^1\)\(^,\)\(^3\) In our earlier studies to explore how this toxicity might be attenuated, we demonstrated that the transfer and expression at high levels of the \textit{Escherichia coli} ATase gene \textit{ada} in a murine hematopoietic stem cell line conferred resistance to the toxic effects of methylaing and chloroethylyating agents.\(^5\) In the next stage of this approach, we have now used a human ATase cDNA-containing retrovirus to infect, ex vivo, murine primary BM cells to examine the extent to which ATase expression and protection against toxicity can be achieved in long-term cultures.

MATERIALS AND METHODS

The human ATase cDNA\(^9\) was cloned into the vector, 50-M-X (Laker et al, submitted), which is based on the murine embryonic stem cell virus\(^10\) but containing the U3 region of the myeloproliferative sarcoma virus to produce 50-M-hAT (Fig 1). This was used to generate amphotrophic virus producers by introduction to GP + envAm2 packaging cells\(^12\) as described.\(^13\) The packaging cells were shown to have incorporated and expressed the 50-M-hAT vector by immunostaining of agarose embedded formalin fixed cells using antihuman ATase polyclonal antibodies (Fig 2).

Long-term cultures from the BM of young adult C57Bl/6 \times DBA2 mice were established as described\(^14\) using 120 \times 10\(^6\) cells in 175-cm\(^2\) tissue culture flasks in Iscove's medium supplemented with 20% horse serum and 10\(^{-6}\) mol/L hydrocortisone. On each of days 0 through 14, the cultures were exposed to approximately 10\(^4\) retrovirus particles by addition of 0.25 vol of virus-containing supernatant from the packaging cells grown in Iscove's medium supplemented with 10% heat-inactivated newborn calf serum. As controls, long-term BM cultures were set up as above and "mock" infected

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Fig 1. Schematic structure of the retroviral vector 50-M-hAT. The 50-M vector was designed to give high expression in early hematopoietic cells by incorporating features of the myeloproliferative sarcoma virus (MPSV) and the murine embryonic stem cell virus (MESV) previously shown to increase transcription efficiency in both embryonic and hematopoietic cells. The cDNA for human 04-alkylguanine-DNA alkyltransferase (hAT) was inserted into a unique BamH1 site. Transcription in infected cells is driven by the MPSV long terminal repeat (LTR).

by addition of fresh culture medium using the same schedule as for virus-infected cultures. During this period, the long-term cultures were supplemented with 10% (vol/vol) of conditioned medium from the 5637 human bladder carcinoma cell line and with 5% (vol/vol) of conditioned medium from murine interleukin-3 (mIL-3) producing myeloma cell line. Nonadherent cells were obtained from long-term cultures every 14 days and the number of granulocyte-macrophage progenitor cells (CFU-GM) was determined by growth in semisolid cultures containing Iscove's medium, 20% horse serum, 5% mIL3 conditioned medium, and 0.3% agar. The sensitivity of progenitor cells to N-methyl-N-nitrosourea (MNU) was determined in agar cultures containing 0 to 100 μg/mL MNU. LD50 dose was determined by dose-response fitting. The sensitivity of long-term hematopoiesis to MNU was determined by repeated administration of drug to the cultures at a concentration of 20 μg/mL on days 36, 43, and 50.

The activity of ATase in the cell extract was determined by quantitation of the transfer of [3H]-methyl groups from [3H]-MNU-methylated calf thymus substrate DNA to the ATase protein in cell extracts as described previously.

The presence of the transduced gene in cells derived from long-term cultures was determined by polymerase chain reaction (PCR) analysis of cell extracts prepared in Taq reaction buffer (Boehringer, Mannheim, Germany) supplemented with 0.45% Tween 20 and 0.5 mg/mL proteinase K using vector-specific (bases 1534-1555 of pM50) and hAT-specific (reverse complement of bases 293-315) primers. PCR conditions were 94°C, 3 minutes; 55°C, 3 minutes; 72°C, 3 minutes for 1 cycle followed by 94°C, 1 minute; 55°C, 1 minute; 72°C, 1.5 minutes, with a final cycle of 72°C, 5 minutes. Products were separated on 2% agarose gels and visualized by ethidium bromide staining.

RESULTS

The presence of the transduced gene in cells derived from virus-exposed and control long-term BM cultures was investigated by PCR using a combination of viral and hAT-specific primers. After PCR analysis of control cell extracts, no hAT-specific PCR products were detected (Fig 3, lanes B and C). In comparison, PCR of virus-exposed cells generated the expected 373-bp product (Fig 3, lane A), demonstrating effective gene transfer.

The mean ATase activity in vector-transduced nonadherent cells obtained from long-term cultures attained a maximum of 5,059 ± 412 fmol/mg protein at day 50 of culture. This activity was more than 70-fold higher than in fresh mouse BM (70 fmol/mg protein) and 1,000-fold higher than in control cultures.
and management of neoplastic disease. The former results in limitations on the tolerated doses of these antitumor agents and the latter results in the development of secondary malignancies, principally leukaemias, in long-term survivors of primary disease. Dose-limiting toxicities can be overcome to some extent by the use of hematopoietic growth factors and/or peripheral blood progenitor cells (PBPCs) mobilized by a combination of growth factors and chemotherapy. However, because many chemotherapeutic regimes involve multiple cycles of treatment, often with dose intensification, growth factors or PBPCs would need to be administered after each round of treatment. Furthermore, neither of these alternative approaches addresses the problems of secondary neoplasms. Indeed, PBPCs are currently mobilized using genotoxic agents, which could lead to an increased incidence of iatrogenic cancers because of DNA damage. For these reasons we have been assessing an alternative approach to overcoming hematotoxicity, using gene transfer of a resistance factor into BM cells.

The sensitivity of hematopoietic cells to the toxic, mutagenic, and clastogenic effects of $O_6$ alkylating agents correlates with their low levels of the specific repair protein, ATase-MEDIATED PROTECTION OF BONE MARROW

![Graph showing Alkyltransferase activity in control and hAT transduced cells.](Fig 4)

**Fig 4.** Mean alkyltransferase activity ± SD (n = 5), in nonadherent hematopoietic cells harvested from long-term BM cultures at day 50 of culture. Control, control cells; hAT transduced, cells transduced with the 50-M-hAT retrovirus. (Fig 4). ATase activity in transduced cells remained elevated in the long-term cultures: although there was some decrease with time, it was still 140 times higher (700 fmol/mg protein) than in control cells at 4 months after transduction.

These levels of ATase clearly provided protection against the toxic effects of MNU: hematopoietic progenitors from the cultures transduced with the hAT retrovirus were three times more resistant to MNU than the progenitors present in control cultures (Fig 5). Furthermore, expression of the human ATase protected long-term hematopoiesis against the cytotoxic effects of MNU: BM cultures were repeatedly exposed to MNU at 20 μg/mL and hematopoietic activity was measured as the output of progenitor cells to the nonadherent fraction of the culture. In control cultures the number of progenitor cells declined steadily after the exposure to MNU (Fig 6A). In the cultures transduced with hAT vector (Fig 6B) the output of progenitor cells was much less affected by the treatment with MNU and, after a slight decrease, within 4 weeks the progenitor cell number recovered to the level seen in cultures not exposed to MNU. Significantly, this long-term protection was achieved against three sequential doses of the nitrosourea.

**DISCUSSION**

The acute and long-term hematotoxicities of $O_6$ alkylating agents present significant clinical problems in the treatment

![Graph showing Resistance to N-methyl-N-nitrosourea of hematopoietic progenitor cells (CFU-GM) in the nonadherent fraction of long-term BM cultures at day 50 of culture.](Fig 5)

**Fig 5.** Resistance to N-methyl-N-nitrosourea of hematopoietic progenitor cells (CFU-GM) in the nonadherent fraction of long-term BM cultures at day 50 of culture. LD₃₇ ± SD (n = 3) of MNU toxicity in mock-transduced (control) hAT-transduced cells.
We have previously shown that the transfer and expression of ATase in hematopoietic cell lines decreases their sensitivity to the effects of alkylating agents. Furthermore, transgenic mice expressing the ATase protein are less susceptible to tumorigenesis subsequent to challenge with alkylating agents.

We now show that expression of the ATase gene product in the hematopoietic cells of long-term BM cultures clearly attenuates the sensitivity of these cells to nitrosoureas and provides long-term protection of hematopoiesis against the cytotoxic effects of these agents. The observation that hematopoiesis may be protected against multiple doses of an agent is of particular interest in a clinical setting where the use of multiple and perhaps escalating doses of drug may be required to achieve remission from disease. In this respect it is particularly important that the appropriate target cells, namely the hematopoietic stem cells, are transduced and protected from the effects of alkylating agents. Protection of committed progenitors would provide only short-term protection against acute toxicities, and after subsequent exposure to chemotherapy, may provide no protection due to clonal extinction of the transduced cells. Furthermore, protection against the longer-term, carcinogenic effects of nitrosoureas can only be achieved if the hematopoietic stem cells are induced to repair the promutagenic genomic damage incurred during antitumor treatment.

Our data show that a human ATase cDNA-containing retrovirus can be used to infect primary BM cultures and direct repair protein expression to levels that are sufficient to provide long-term protection against the toxic effects of alkylating agents. Expression of ATase is maintained for the hematopoietic life of the cultures (22 weeks). In the long-term BM culture system, the input committed progenitor cells undergo differentiation and clonal extinction within the first 6 to 9 weeks of culture. Therefore, continuation of gene expression in cells produced by these cultures for 20 weeks post gene transduction implies that primitive, long-term culture-initiating cells have been transduced and that the gene is expressed in their progeny.

While this work was under review, two complementary studies have been published that underline the efficacy of this approach. In these studies, short-term protection of in vitro and in vivo hematopoiesis against the cytotoxic effects of single doses of nitrosoureas was shown after retroviral transfer of the ATase cDNA to mouse BM cells. Our demonstration of long-term expression of ATase and protection of hematopoiesis, taken with the recent in vivo studies, suggests that stem cell protection using ATase gene transfer may be feasible. However, before any progression to human in vivo experiments can occur it is important that extensive long-term studies in experimental animals be performed in which the effects of ATase expression in BM on the collateral toxicity of widely used methylating and related agents is fully investigated. Furthermore, it will be important to determine what, if any, effect ATase expression will have on the longer-term (carcinogenic) side effects of nitrosourea treatment.

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Long-term protection of hematopoiesis against the cytotoxic effects of multiple doses of nitrosourea by retrovirus-mediated expression of human O6-alkylguanine-DNA-alkyltransferase

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